

International Comparison of Clinical, Bovine, and Environmental *Escherichia coli* O157 Isolates on the Basis of Shiga Toxin-Encoding Bacteriophage Insertion Site Genotypes[∇]

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***Escherichia coli* O157:H7 genotypes in the bovine reservoir may differ in virulence. The proportion of clinical genotypes among cattle isolates was weakly ($P = 0.054$) related to the international incidence of *E. coli* O157:H7-associated hemolytic-uremic syndrome, varied among clinical isolates internationally, and also differed along the putative cattle-hamburger-clinical case transmission chain.**

Infection with enterohemorrhagic *Escherichia coli* serotypes O157:H7 and O157:H– (EHEC-O157) may cause diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (13, 24). Cattle are considered the principal reservoir of EHEC (18). EHEC-O157 typically produces Shiga toxins Stx1 and/or Stx2, encoded by lambdoid bacteriophages (23, 28, 34). In EHEC-O157 strains EDL933 and Sakai, Stx-encoding phages are inserted in *yehV* and *wrbA* (19, 40). Shaikh and Tarr (44), however, demonstrated insertion site diversity in Stx-encoding bacteriophages among clinical EHEC-O157 isolates, defining three predominant clinical genotypes (genotypes 1 to 3). Subsequently, the predominance of genotypes 1 to 3 among a larger set of U.S. human clinical isolates was confirmed; in contrast, considerable additional diversity of Stx-encoding bacteriophage insertion sites was demonstrated in isolates from the bovine reservoir (6). Since nonclinical genotypes represented almost half of the bovine isolates, broad exposure of the human population to these genotypes would be expected.

The frequency of reported EHEC-O157-associated disease varies markedly internationally. For example, the incidence of EHEC-O157 (infections/100,000 population annually) was reported as 4.1 (Scotland, 2004), 0.9 (United States, 2004), 0.87 (Japan, 2004), 0.13 and 1.6 (Germany, 2004 and 1997 to 2003, respectively), 0.11 (Republic of Korea, 2003), and 0.08 (Australia, 2004) (2, 9, 14, 15, 27, 31, 38). HUS, an uncommon sequel to EHEC-O157 infection, may be less underreported due to its severity (32). The corresponding incidence of EHEC-O157-associated HUS was 0.41 (Scotland), 0.1 (United States), 0.002 to 0.20 (Germany), 0.05 (Republic

of Korea), and 0.01 (Japan and Australia) (1, 2, 10, 14, 15, 20, 37).

To determine whether the proportion of EHEC-O157 genotypes in the bovine reservoirs influences the rates of the diverse international incidence of EHEC-O157 disease, we genotyped EHEC-O157 isolates obtained from cattle in several countries. Study isolates included non-sorbitol-fermenting, β -glucuronidase-negative EHEC-O157 isolates from cattle originating from different farms in geographically disseminated locations within the United States (1994 to 2002), Australia (1993 to 2003), Japan (1996 to 1997; provided by Masato Akiba, National Institute of Animal Health, Tsukuba, Ibaraki, Japan), Scotland (1999; provided by Barti Synge, Scottish Agricultural College, Inverness, United Kingdom), and Korea (1997; provided by B. Young).

Genotypes of EHEC-O157 isolates were determined by using a multiplexed variation of a PCR method previously described (6, 44). Multiplex 1 included *stx*₁ (36), the right *wrbA*-bacteriophage junction, and the left *yehV*-bacteriophage junction. Multiplex 2 included *stx*₂ (39), the left *wrbA*-bacteriophage junction, and the right *yehV*-bacteriophage junction. EHEC-O157 cells were grown overnight at 37°C in LB broth with shaking and diluted 1:10 with water for use as a whole-cell template. The 50- μ l reaction mixtures included 2.5 U/ μ l *Taq* polymerase, 2 mM MgCl₂, 0.4 mM deoxynucleoside triphosphates, 5 μ l 10 \times buffer (Invitrogen, Carlsbad, CA), and 2 μ l of the whole-cell template. Thermocycler (iCycler; Bio-Rad, Hercules, CA) parameters included one 95°C (5 min) cycle and 35 cycles at 94°C (30 s), 58°C (45 s), and 72°C (90 s), followed by a final 72°C (10 min) cycle. The assignment of genotypes was based on the presence or absence of the six PCR products (6). Controls included *E. coli* DH5 α (negative control) and EDL933 (positive control).

No significant association was observed between the proportion of clinical genotypes among isolates from the international bovine reservoirs and the respective international incidences of EHEC-O157 disease (r_s [Spearman's rho statistic] = 0.50, $P =$

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TABLE 1. Genotypes of Stx-encoding bacteriophage insertion sites from an international group of clinical, bovine, and environmental isolates of EHEC-O157

Genotype ^a	Presence or absence of PCR products ^b	No. (%) ^c of isolates from indicated country and source:											
		Australia		Japan		Germany		Korea	Scotland	United States ^d			
		Bovine	Human	Bovine	Human	Human ^c	Human	Bovine	Bovine	Bovine	Beef	Human	Sewage
1	011100	21 (35.0) ^e	3 (20.0)	8 (18.2)	1 (20.0)	0 (0)	21 (72.4)	4 (12.9)	22 (56.4)	22 (15.3)	18 (17.5)	39 (26.4)	8 (66.7)
2	011111	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.4)	2 (6.5)	0 (0)	2 (1.4)	5 (4.9)	4 (2.7)	0 (0)
3	111111	2 (3.3)	4 (26.7)	8 (18.2)	2 (40.0)	0 (0)	0 (0)	8 (25.8)	0 (0)	31 (21.5)	35 (34)	82 (55.4)	0 (0)
4	010011	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (2.1)	0 (0)	1 (0.7)	0 (0)
5	011000	2 (3.3)	2 (13.3)	14 (31.8)	1 (20.0)	0 (0)	4 (13.8)	8 (25.8)	2 (5.1)	46 (31.9)	10 (9.7)	4 (2.7)	1 (8.3)
6	111000	15 (25.0)	2 (13.3)	3 (6.8)	0 (0)	0 (0)	0 (0)	0 (0)	7 (17.9)	31 (21.5)	10 (9.7)	3 (2.0)	0 (0)
7	111011	0 (0)	0 (0)	1 (2.3)	1 (20.0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	0 (0)	1 (0.7)	0 (0)
8	101100	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (9.7)	0 (0)	0 (0)	3 (2.9)	2 (1.4)	0 (0)
9	000000	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (3.2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
10	001100	0 (0)	0 (0)	1 (2.3)	0 (0)	0 (0)	0 (0)	1 (3.2)	3 (7.7)	1 (0.7)	2 (1.9)	1 (0.7)	2 (16.7)
11	010000	0 (0)	0 (0)	0 (0)	0 (0)	29 (100)	1 (3.4)	1 (3.2)	0 (0)	0 (0)	1 (1)	1 (0.7)	0 (0)
12	011011	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (1)	1 (0.7)	0 (0)
13	101000	1 (1.7)	2 (13.3)	2 (4.5)	0 (0)	0 (0)	0 (0)	0 (0)	3 (7.7)	1 (0.7)	1 (1)	2 (1.4)	0 (0)
14	101011	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
15	110000	5 (8.3)	2 (13.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.4)	1 (1)	0 (0)	0 (0)
16	111100	12 (20.0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (6.9)	1 (3.2)	0 (0)	3 (2.1)	8 (7.8)	4 (2.7)	0 (0)
17	001000	0 (0)	0 (0)	1 (2.3)	0 (0)	0 (0)	0 (0)	2 (6.5)	2 (5.1)	0 (0)	0 (0)	0 (0)	0 (0)
18	111101	0 (0)	0 (0)	6 (13.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.9)	1 (0.7)	0 (0)
19	010100	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (3.9)	0 (0)	1 (8.3)
20	100000	2 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	0 (0)
21	011101	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.9)	0 (0)	0 (0)
22	101111	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	0 (0)
23	111110	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.7)	0 (0)	0 (0)	0 (0)
Total		60	15	44	5	29	29	31	39	144	103	148	12

^a Genotypes 1 to 3 (clusters 1 to 3) (44) and 4 to 16 (6).^b Presence or absence of PCR products for *stx*₁, *stx*₂, *yehV*-left, *yehV*-right, *wrbA*-left, and *wrbA*-right shown in concatenated code. 1, present; 0, absent.^c Sorbitol-fermenting, β-glucuronidase-positive EHEC-O157:H-; all other columns are for non-sorbitol-fermenting, β-glucuronidase-negative *Escherichia coli* O157:H7.^d Beef, retail ground beef; sewage, untreated municipal sewage.^e Percentage of total number of isolates for the column.

0.39) (Table 1). In contrast, the correlation between the proportion of clinical genotypes in the bovine reservoirs and the respective international incidences of HUS approached statistical significance ($r_s = 0.87$, $P = 0.054$). Isolates from Scottish cattle had the highest proportion of clinical genotypes 1 to 3

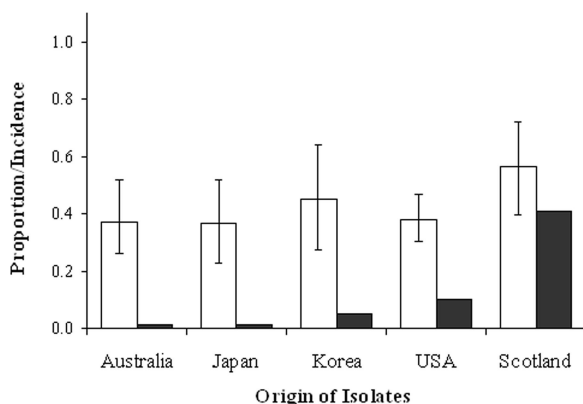


FIG. 1. The proportion of bovine isolates with clinical genotypes (genotypes 1 to 3, unfilled bars) among isolates from cattle in the specified countries and the incidence (cases per 100,000 population, filled bars) of EHEC-O157 HUS are shown. Error bars indicate 95% confidence intervals.

(56%) (Fig. 1), but the relative numbers of clinical genotypes in cattle isolates in the United States (38%), Korea (45%), Australia (37%), Japan (36%), and Scotland did not differ ($\chi^2 = 5.1$; 4 df; $P = 0.28$). Therefore, the effect of EHEC-O157 genotypes would appear to be limited to, at most, the more-severe disease manifestation of HUS. Even for HUS, the observed ~2-fold difference in the proportions of clinical genotypes in isolates from the bovine reservoir is far smaller than the 40-fold difference in the reported incidences of EHEC-O157-associated HUS, suggesting that other factors must account for most of the international differences. Such factors may include differences in the magnitudes of shedding of EHEC-O157 for specific genotypes or differing international prevalences of EHEC-O157 shedding by cattle. Reports of bovine prevalence vary widely, both within and between countries, in part as a result of different sampling, culture, and isolation methods used for EHEC-O157 detection (11, 12, 16, 21, 22, 30, 33, 35, 42, 43, 45). International comparisons of the prevalence of cattle infection, the magnitudes of cattle fecal shedding, and the frequency of contamination of human food and water sources using standardized methods and sampling frames, in conjunction with genotype determinations of the isolates in those sources, would be required to more accurately address the effects of the EHEC-O157 genotypes present in bovine reservoirs on the incidence of human disease.

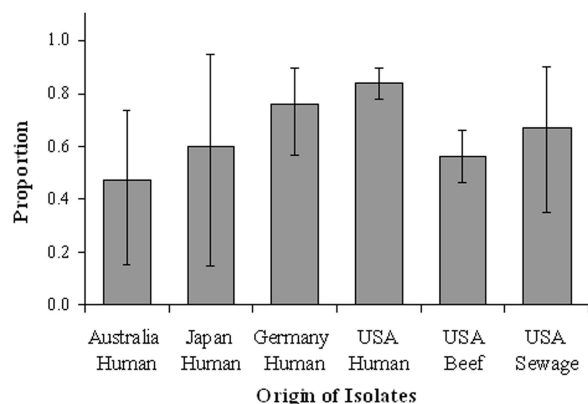


FIG. 2. The proportion of clinical genotypes (genotypes 1 to 3) in human clinical isolates and isolates from retail ground beef and untreated municipal sewage in the specified countries. Error bars indicate 95% confidence intervals.

Where available to us, we also analyzed clinical EHEC-O157 isolates to determine if the relative prevalences of clinical genotypes are similar internationally. Clinical isolates were obtained from the United States (2004 to 2005; Washington Department of Health), Japan (1995 to 1996; M. Akiba), Australia (1986 to 1999; R. Robins-Browne and D. Lightfoot, University of Melbourne, Parkville, Victoria, Australia), and Germany (DNA from both sorbitol-fermenting and non-sorbitol-fermenting isolates; Martina Bielaszewska, University of Münster, Münster, Germany). Since DNA from all of the sorbitol-fermenting, β -glucuronidase-positive EHEC-O157 German isolates were negative for all Stx insertion site genotyping markers except *stx*₂, consistent with its status as a distinct clade of EHEC-O157, the following analyses of genotypes were limited to isolates of the non-sorbitol-fermenting clade.

The proportions of clinical genotypes differed significantly among clinical isolates obtained from different countries ($\chi^2 = 13.7$; 3 df; $P < 0.005$), including 84% (United States), 76% (Germany), 60% (Japan), and 47% (Australia). With Bonferroni's correction, pairwise analyses demonstrated that the proportions of clinical genotypes among clinical isolates differed significantly between the United States and Australia only ($P < 0.02$).

Lastly, we compared genotypes of EHEC-O157 strains isolated from along the putative transmission chain from cattle, retail ground beef, clinically ill humans, and untreated sewage. Ground beef isolates were provided by Marcus Head, United States Department of Agriculture Food Safety and Inspection Service, Athens, Georgia. Additional isolates were obtained from untreated sewage at two municipal sewage treatment facilities in Washington State in 2006. The proportions of clinical genotypes were significantly higher among clinical isolates than from cattle or ground beef specimens ($P < 0.01$) (Fig. 2), but surprisingly different proportions of clinical genotypes were observed in isolates from cattle feces and from retail ground beef specimens ($\chi^2 = 7.9$; 1 df; $P < 0.01$) (Table 1 and Fig. 1 and 2), with relatively more genotype 3 isolates, and smaller amounts of genotype 5 and 6 isolates from ground beef than from cattle feces. These differences may be due to differential fitness among some genotypes, such as higher shedding

levels in cattle feces, increased ability to survive processing and persist on hamburger and other food products, or other similar traits. Strain-specific differences in survival on beef or in media (3, 4, 5, 7, 41) have been reported, some in the opposite direction (4) from the tendency to explain the differences in genotypes reported here.

Differences in EHEC-O157 genotypes among clinical and bovine reservoir isolates have been previously reported, including those detected by Octamer Based Genomic Scanning (OBGS; lineage I versus lineage II), with the bacteriophage antiterminator allele Q933 (presence versus absence) and a polymorphism in *tir* (255 T versus A), and by phage typing (21/28 versus others from Scotland) (8, 17, 25, 26, 29, 31). Some of these genotypes may be correlated; for example, both Stx insertion typing and OBGS classify most Australian isolates into genotypes less associated with clinical disease (lineage II and nonclinical genotypes, respectively). The biologic basis of the differential representation of these genotypes in cattle and in human disease remains largely unexplained.

In summary, while the proportion of clinical genotypes in the bovine reservoir tended to correlate with the incidence of HUS in human populations, this tendency was too weak to provide a satisfying explanation for the magnitude of the differences in the international incidences of HUS and other EHEC-O157-related diseases. Assuming that the incidence estimates for EHEC-O157 disease are accurate, then other factors, such as the prevalence of EHEC-O157 in cattle, genotype-related differences in fecal shedding by cattle, survival in food products and environmental niches, and infectivity and virulence, as well as differences in food preparation practices and dietary composition, may contribute significantly to the differing international incidences of EHEC-O157 disease.

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